

# Physiological expression of miR-130a during differentiation of CD34<sup>+</sup> human hematopoietic stem cells results in the inhibition of monocyte differentiation

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## ABSTRACT

MicroRNAs (miRNA) are small noncoding RNAs that regulate gene expression by targeting mRNAs in a sequence specific manner, thereby determining their degradation or inhibiting translation. They are involved in processes such as proliferation, differentiation and apoptosis by fine-tuning the expression of genes underlying such events. The expression of specific miRNAs is involved in hematopoietic differentiation and their deregulation contributes to the development of hematopoietic malignancies such as acute myeloid leukemia (AML). miR-130a is over-expressed in AML. Here we show that miR-130a is physiologically expressed in myeloblasts and down-regulated during monocyte differentiation. Gain- and loss-of-function experiments performed on CD34<sup>+</sup> human hematopoietic stem cells confirmed that expression of miR-130a inhibits monocyte differentiation by interfering with the expression of key transcription factors HOXA10, IRF8, KLF4, MAFB and PU-1. The data obtained in this study highlight that the correct modulation of miR-130a is necessary for normal differentiation to occur and confirming that deregulation of this miRNA might underlie the differentiation block occurring in AML.

## 1. Introduction

miRNAs represent an additional regulatory mechanism in hematopoietic stem cells (HSCs) by influencing transcription profiles and transcript stability [1]. Transcription factors (TF) and miRNAs act in concert to regulate gene expression during hematopoietic differentiation; TFs regulate the expression of miRNA genes, whereas TFs are key miRNA targets. Thus, miRNAs represent another axis of regulation in HSCs, controlling the self-renewal and the fine-tuning of cell fate during the lineage-specification process. Numerous studies have identified

more than 100 different miRNAs specifically expressed during hematopoiesis in mice and humans [2]. In addition, two studies have shown impaired hematopoiesis after the conditional deletion of Dicer and Argonaute2, demonstrating the functional impact of miRNAs on normal hematopoietic process [2–4].

Several miRNAs appear to influence the commitment of HSCs and their progenitors to monocyte. For instance, miR-146a, miR-155, miR-342 and miR-338 are upregulated by transcription factor PU.1 [5,6], which controls myeloid cell development, and ectopic expression of miR-146a is sufficient to direct HSC differentiation to the mononuclear

**Abbreviations:** Acute myeloid leukemia, AML; Hematopoietic stem cells, HSC; Transcription Factor, TF; Vitamin D, VD; Peptide nucleic acid, PNA; Cord blood, CB; Flt3-ligand, Flt3-l; Real Time Quantitative PCR, RTQPCR; Colony forming units granulocyte/monocyte, CFU-GM; Colony forming units monocyte, CFU-M; Myeloperoxidase, MPO

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phagocyte lineage in mouse transplantation assays [5]. miR-130a is aberrantly over-expressed in AML [7], and M1 AML compared to M5 AML and directly targets transcription factors MAFB, IRF8 and KLF4 [8] that are determinants of monocyte differentiation.

On these grounds, we evaluated how expression of miR-130a is modulated through myeloid differentiation and in response to a monocyte differentiation inducer such as vitamin D (VD). Results show that the selected miRNAs are up-regulated in myeloblasts, compared to HSCs, while their expression is suppressed in the monocyte lineage and in HSCs stimulated with VD. To gain further insight on the biological meaning of these preliminary observations, we performed gain- and loss-of-function experiments in HSCs. Loss of function experiments were performed with two different approaches: by transfecting HSCs with anti-miRNA oligonucleotides or with Peptide Nucleic Acids (PNA) targeting miRNA. PNAs are DNA analogs in which the sugarphosphate backbone is replaced by N-(2-aminoethyl) glycine units [9]. These molecules were described for the first time by Nielsen et al. [10] and, despite a radical structural difference with respect to DNA and RNA, they are capable of sequence-specific and efficient hybridization with complementary DNA and RNA, forming Watson-Crick double helices [11,12]. The efficacy of PNAs in targeting miRNAs was demonstrated by several works [13–16]. Gain- and loss-of-function assays performed in HSCs confirmed that expression of miR-130a is tightly related to monocyte differentiation where, in particular, miRNA's over-expression inhibits monocyte differentiation, while miRNA's silencing favors it.

## 2. Materials and methods

### 2.1. Ethics statement

Human CD34<sup>+</sup> cells were purified upon donor's informed written consent from healthy donor-derived umbilical Cord Blood (CB) samples, collected after normal deliveries, according to the institutional guidelines for discarded material (Clearance of Ethical Committee for Human experimentation of Modena: Secretary office Saverio Santachiara, [santachiara.saverio@policlinico.mo.it](mailto:santachiara.saverio@policlinico.mo.it), approval date: 18 January 2005; approval file number # 793/CE).

### 2.2. Cell population

Cord Blood (CB) CD34<sup>+</sup> cells were purified as previously described [17]. After immunomagnetic separation, CD34<sup>+</sup> cells were cultured in IMDM (Euroclone s.p.a., Milan, Italy) supplemented with 20% FCS (Bio-Whittaker, Walkersville, MD, USA), in the presence of human hematopoietic cytokines: SCF (50 ng/ml), Flt3-ligand (Flt3-l) (50 ng/ml), IL-11 (50 ng/ml), IL-6 (10 ng/ml), IL-3 (10 ng/ml) and G-CSF (10 ng/ml) (all from R&D Systems, Minneapolis, MN, USA) [17].

Monoblasts and Myeloblast derived from a 7 days culture of CD34<sup>+</sup> were separated using the EasySep Human CD14-Positive Selection Kit (Stemcell Technologies, Vancouver, Canada) [18]. The same method is used to collect monocytes from adult peripheral blood mononuclear cells.

Human granulocytes were collected from cell pellets obtained by Ficoll separation of PBMCs. Erythrocytes contained in cell pellets were removed by means of osmotic lysis. Neutrophils (CD16<sup>+</sup> fraction) were then purified using the EasySep Human "Do-It-Yourself" Selection Kit (Stemcell Technologies) conjugated to mouse Mo anti-human CD16 Ab (Miltenyi, Auburn, CA) [19].

CD34<sup>+</sup> cells, cultured in the same described above conditions, were stimulate for 7 days with Vitamin D (VD) (Hoffman-Laroche) at  $5 \times 10^{-8}$  M concentration [17].

### 2.3. RNA extraction

Total cellular RNA, including small RNAs, was isolated using the miRvana miRNA Isolation kit (Thermo Fisher Scientific, Austin, TX,

USA) following the manufacturer's protocol.

For each sample RNA concentration and integrity were assessed by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Wilmington).

### 2.4. miRNA precursor molecule and Anti-miRNA inhibitor transfections

**miRNA precursor molecule transfections.** After separation, CD34<sup>+</sup> cells were cultured in the conditions previously described [19]. After 2 days of culture, the cells were transfected with the Amaxa Nucleofector Device, using the Human CD34 Cell Nucleofection Kit, according to the manufacturer's instructions (Lonza, Basil, Switzerland). 5 µg of the following mirVana miRNA mimic, Negative Control #1, miR-130a-3p, (Thermo Fisher Scientific) were pulsed with the program U-008.

Pre-miR Negative Control #1 is a random sequence miRNA precursor molecule that has been extensively tested in human cell lines and tissues and validated to not produce identifiable effects on known miRNA function (<http://www.ambion.com>).

**Anti-miRNA inhibitor transfections.** After separation, CD34<sup>+</sup> cells were cultured in the conditions previously described. After 2 days of culture, they were transfected with the Amaxa Nucleofector Device, using the Human CD34 Cell Nucleofection Kit, according to the manufacturer's instructions (Amata Biosystem). 5 µg of the following mirVana miRNA inhibitors, Negative Control #1, miR-130a (Thermo Fisher Scientific) was pulsed with the program U-008. Anti-miR Negative Control #1 is a random sequence anti-miRNA inhibitor molecule that has been extensively tested in human cell lines and tissues and validated to not produce identifiable effects on known miRNA function (<http://www.ambion.com>).

### 2.5. Quantitative RT-PCR

QRT-PCR was carried out by the ABI PRISM 7900 sequence detection system (Thermo Fisher Scientific) on total RNAs (100 ng) reverse transcribed using the High Capacity cDNA Archive Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Each cDNA sample was run in triplicate for targets (MAFB, MMP9, IL-7R, IL-6, TNF-α, IL-1RA, MRC1, CD163, CD14, CCL2, IL8, IL10Rβ, CEBPβ, GYPa, MPO) and for GAPDH endogenous control. Thermo Fisher Scientific supplied primers and probes and the TaqMan Universal Master Mix. Quantification of QRT-PCR signals was performed using the (2<sup>-ΔΔCt</sup>) method, as previously described [20]. The values obtained were represented in terms of relative quantity of mRNA level variations.

miRNAs was reverse transcribed and TaqMan reactions were carried out using the TaqMan MicroRNA Assays Kit. PCR reactions were performed using TaqMan Universal PCR Master Mix without AmpErase Uracil N-glycosylase, by means of the ABI PRISM 7900 HT Sequence Detection Systems (all from Thermo Fisher Scientific).

ΔΔ-CTs and Relative Quantity were calculated, for each detector using as calibrator the CD34<sup>+</sup> sample and the TaqMan Control miRNA Assay RNU6b as endogenous control.

### 2.6. CFC assay

After 24 h from the nucleofections, CD34<sup>+</sup> cells were plated following the manufacturer's instructions in MethoCult GF H4434 complete methylcellulose medium for clonogenic assay (StemCell Technologies) containing a cocktail of recombinant human cytokines: SCF (50 ng/ml), granulocyte-macrophage colony-stimulating factor (10 ng/ml), interleukin-3 (10 ng/ml) and EPO (3 U/ml). After 14 days of culture at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>, colonies were scored as previously described [21].

## 2.7. Protein extract preparation and Western blot analysis

Total protein extracts were obtained using a small volume of lysis buffer (as previously described [22]). 30 µg of extracted protein was then loaded onto 10% SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose sheets. Blotted membranes were pre-blocked for 1 h at room temperature (RT) in blocking solution, composed by 5% nonfat milk (Regilait, Saint-Martin-Belle-Roche, France) in 0.05% TBST or by 3% nonfat milk and 2% BSA (Sigma Aldrich, St. Louis, MO, USA) in TBST 0.1%, according to antibodies specificity.

The following rabbit anti-human primary antibodies were used at concentrations recommended by the manufacturer's instruction: MafB polyclonal Ab (Sigma-Aldrich), Klf4 (Abcam, Cambridge, UK), Irf8 and Hox10 (Santa Cruz Biotechnology, Dallas, Texas, USA).

As a secondary antibody, conjugated to horse-radish peroxidase, we used a goat anti-rabbit IgG (Cell Signaling Technology) at 1:3000 dilutions. To normalize analyzed protein samples, a rabbit anti-human vinculin polyclonal Ab (Millipore Corporation, Billerica, MA, USA) were used. Detection of Western blot signals was carried out using the Westar Etc enhanced chemiluminescent substrate (Cyanagen S.r.l., Bologna, Italy).

## 2.8. Morphological and immunophenotypic analysis

Differentiation of CD34<sup>+</sup> cells was assessed by morphological analysis with May-Grunwald-Giemsa staining and by flow-cytometric analysis of differentiation markers expression 8 day after the nucleofection.

The following monoclonal antibodies (MoAbs) were used for flow cytometric analysis: fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD14 MoAb, phycoerythrin (PE)-conjugated mouse anti-human CD163 MoAb (all from Miltenyi Biotec, Bergisch Gladbach, Germany). Analysis was performed in terms of positivity percentage with the Coulter Epics XL-MCL (Coulter Electronics, Hialeah, FL) flow cytometer as previously described [23]. At least 10,000 events for sample were acquired.

## 2.9. Peptide nucleic acids synthesis

If not otherwise mentioned, chemicals and solvents were purchased from Sigma Aldrich, Alfa Aesar, or Scharlab, and used without any further purification. DMF was dried over 4 Å molecular sieves and purged with nitrogen to avoid the presence of dimethylamine.

The synthesis and characterization of PNAs was similar to those previously reported [24]. The synthesis was performed using standard Fmoc-based automatic peptide synthesizer (Syro I, MultiSynTech GmbH, Witten, Germany), using a ChemMatrix-RinkAmide resin loaded with Fmoc-Gly-OH (0.2 mmol/g) as first monomer and using commercially available Fmoc/Bhoc PNA monomers (Link Technologies, Bells-hill, UK) and Fmoc-Arg (Pbf)-OH, with HBTU/DIPEA coupling. Cleavage from the solid support was performed with 10% m-cresol in trifluoroacetic acid (TFA), followed by precipitation and washings with diethyl ether. Purification was performed by HPLC using a Jupiter RPC18 column (250 × 4.6 mm, 1.7 µm, Phenomenex, Torrance, Ca, USA). Gradient: 100% A for 5 min, then from 0 to 50% B in 30 min at 4 mL/min flow (A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile). Column temperature: 40 °C. After purification the PNAs were characterized by UPLC-MS or by HPLC-HRMS. For UPLC-MS the following conditions were used: Waters Acquity Ultra Performance LC with Waters SQ detector, and equipped with Waters UPLC BEH 300 column (50 × 2.1 mm, 1.7 µm, C18). A flow rate of 0.25 ml/min was used with the following solvent systems: (A): 0.2% FA in water and (B): 0.2% FA in acetonitrile (FA = formic acid). The column was flushed for 0.9 min with solvent A, then a gradient from 0 to 50% B in 5.7 min was used.

For HPLC-HRMS the following HPLC-MS instrumental set-up was used: DIONEX Ultimate3000 system (Thermo Scientific Waltham, MA,

USA) coupled with a LTQ Orbitrap XL spectrometer (Thermo Scientific); Software: Xcalibur 2.0.7 SP1 (Thermo Fisher Scientific Waltham, MA, USA) Column: AERIS Peptides 3.6 µm XB-C18 150 × 2.1 mm (Phenomenex). Chromatographic condition: eluent A: water + 0.2% formic acid; eluent B: acetonitrile + 0.2% Formic acid. Column temperature: 35 °C. Program: initial isocratic at 10% B (5 min), then linear gradient to 95% B (in 25 min). Flow rate: 0.2 mL/min. The concentrations of the PNAs were calculated using UV-absorbance at 260 nm assuming an additive contribution of all bases ( $\epsilon = 198,000 \text{ M}^{-1} \text{ cm}^{-1}$  for all PNAs). **R8-PNA-a130a**: sequence H-R8-CCTTTTAACATTGCACTG-Gly-NH<sub>2</sub>; yield 8.2%; HPLC-HRMS Rt = 12.47 min, calculated MW: 6127.29 g/mol; m/z found (calculated): 1532.70 (1532.82) [MH<sub>4</sub>]<sup>4+</sup>, 1226.36 (1226.46) [MH<sub>5</sub>]<sup>5+</sup>, 1022.13 (1022.22) [MH<sub>6</sub>]<sup>6+</sup>, 876.26 (876.33) [MH<sub>7</sub>]<sup>7+</sup>, 766.85 (766.91) [MH<sub>8</sub>]<sup>8+</sup>, 681.76 (681.81) [MH<sub>9</sub>]<sup>9+</sup>, 613.68 (613.73) [MH<sub>10</sub>]<sup>10+</sup>. **R8-PNA-MIS-130a**: sequence H-R8-CCATTTTACAATGCTCTG-Gly-NH<sub>2</sub>; yield 8.18%; HPLC-MS Rt = 12.53 min, calculated MW: 6127.29 g/mol; m/z found (calculated): 1532.70 (1532.82) [MH<sub>4</sub>]<sup>4+</sup>, 1226.36 (1226.46) [MH<sub>5</sub>]<sup>5+</sup>, 1022.14 (1022.22) [MH<sub>6</sub>]<sup>6+</sup>, 876.26 (876.33) [MH<sub>7</sub>]<sup>7+</sup>, 766.85 (766.91) [MH<sub>8</sub>]<sup>8+</sup>, 681.76 (681.81) [MH<sub>9</sub>]<sup>9+</sup>, 613.68 (613.73) [MH<sub>10</sub>]<sup>10+</sup>. **R8-PNA-SCR-130a**: sequence H-R8-ATCTCGTATCTATCCTGA-Gly-NH<sub>2</sub>; yield 12.52% (after purification); HPLC-HRMS Rt = 11.95 min, calculated MW: 6127.29 g/mol; m/z found (calculated): 1532.70 (1532.82) [MH<sub>4</sub>]<sup>4+</sup>, 1226.36 (1226.46) [MH<sub>5</sub>]<sup>5+</sup>, 1022.13 (1022.22) [MH<sub>6</sub>]<sup>6+</sup>, 876.26 (876.33) [MH<sub>7</sub>]<sup>7+</sup>, 766.85 (766.91) [MH<sub>8</sub>]<sup>8+</sup>, 681.76 (681.81) [MH<sub>9</sub>]<sup>9+</sup>, 613.68 (613.73) [MH<sub>10</sub>]<sup>10+</sup>, 557.99 (558.03) [MH<sub>11</sub>]<sup>11+</sup>.

## 2.10. PNA transfection

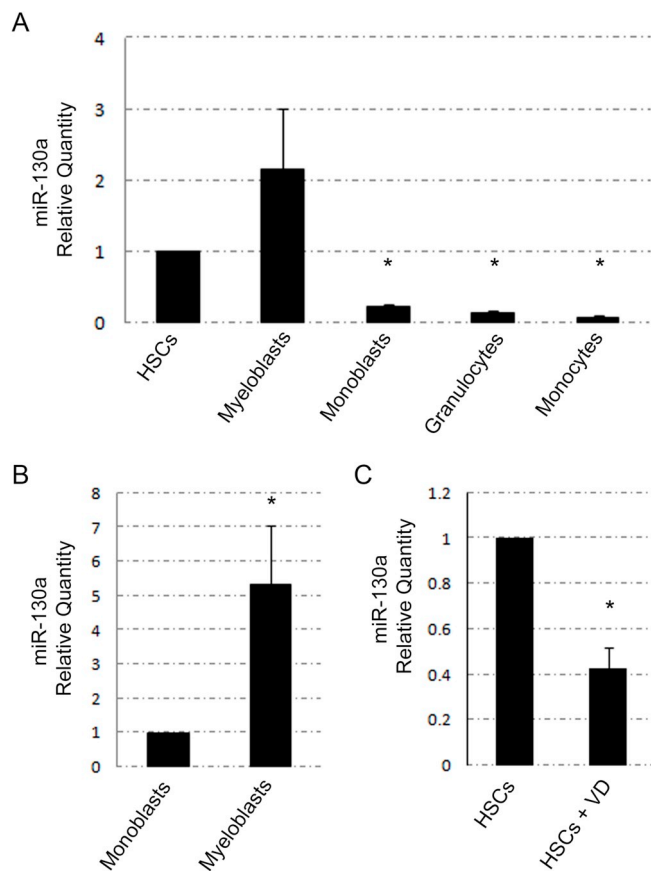
PNA and controls were directly added to the cellular medium at a final concentration of 2 µM in HSCs. Western blots on transcription factors were performed 48 h after treatment, clonogenic assays were counted two weeks following PNA treatment. Cell morphology was assessed a week after treatment.

## 3. Results

**Expression of miR-130a in normal haematopoiesis.** Fig. 1A represents a Real Time quantitative PCR (QRT-PCR) showing the expression level of miR-130A in myeloblasts, monoblasts, granulocytes and monocytes compared to HSCs. Results show that miR-130a is up-regulated in myeloblasts and down-regulated in the other three analyzed cell contexts compared to HSCs. A direct comparison, obtained by QRT-PCR, between the expression of miR-130a in monoblasts and myeloblasts is shown in Fig. 1B, confirming miR-130a down-regulation in monoblasts. Fig. 1C depicts another QRT-PCR showing expression levels of miR-130a in HSCs before and after treatment with the monocyte differentiation inducer vitamin D (VD). This experiment shows that miR-130a is down-regulated upon VD induced differentiation.

**Ectopic expression of miR-130a in human CD34<sup>+</sup> haematopoietic stem cells.** In this set of experiments, HSCs were nucleofected with pre-miR-130a. Effects of ectopic expression were analyzed by different approaches. Fig. 2A summarizes a methylcellulose assay showing that, upon miR-130a ectopic expression, a statistically significant increase in “colony-forming-units granulocyte/monocyte” (CFU-GM) coupled to a decrease of “colony forming units monocyte” (CFU-M) occurs. Fig. 2B represents a QRT-PCR performed on a pattern of monocyte-related genes. Following ectopic expression of miR-130a, there is a general and statistically significant down-regulation of such genes, among whom CD14, CD163, IL1a, IL7R and MMP9 are particularly relevant. Myeloperoxidase (MPO), whose expression does not change, is a granulocyte-related gene that was added as a specificity control.

Fig. 2C summarizes the flow cytometry analysis showing the

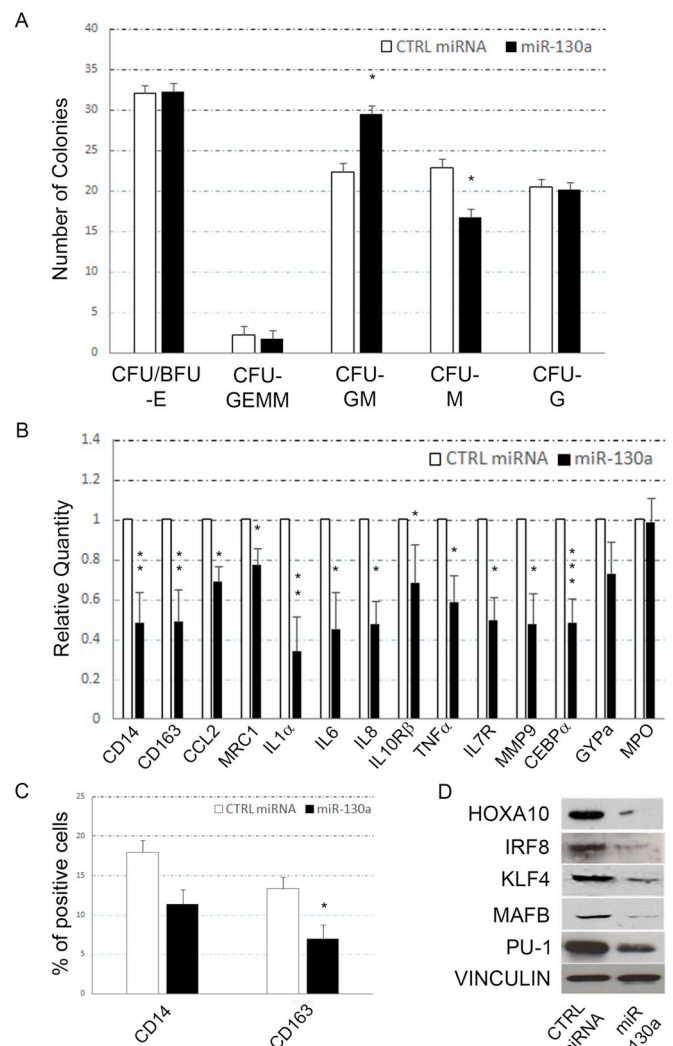


**Fig. 1.** Expression of miR-130a in normal haematopoiesis. (A) QRT-PCR showing that among different myeloid contexts, miR-130a is especially expressed in myeloblasts while it is down-regulated in monoblasts, granulocytes and monocytes. (B) QRT-PCR confirming miR-130a down-regulation in monoblasts. (C) QRT-PCR showing that VD treatment inhibits miR-130a expression in HSCs. In all experiments mean variations of mRNA expression levels, deriving from at least three independent experiments, are reported in the y axis as relative fold-change (relative quantity). Results are represented as mean  $\pm$  S.E.M. values and asterisks indicate statistically significant results ( $P < 0.05$ ).

percentage of cells positive to CD14 and CD163 in untreated HSCs and in cells ectopically expressing miR-130a. Following miRNA expression, a reduction of both CD14 and CD163 positivity is observed. Fig. 2D shows a Western blot analysis of several key transcription factors related to monocyte differentiation. Upon miR-130a over-expression in HSCs, they appear to be generally down-regulated also at the protein level.

**Effect of miR-130a silencing in human CD34<sup>+</sup> haematopoietic stem cells.** HSCs were transfected with Anti miR-130a in order to block its expression and observe the effect on haematopoietic differentiation. Fig. 3A shows that, upon miR-130a silencing, in a methylcellulose assay one can observe a reduction of CFU-GM and an increase of CFU-M, opposite to what was observed in the “gain of function” set of experiments. Fig. 3B represents a QRT-PCR showing that miR-130a silencing in HSCs determines the upregulation of monocyte-related genes (IL7R, MMP9 and MRC1), which is coupled to increased positivity to monocyte markers CD14 and CD163, as demonstrated by flow cytometry analysis (Fig. 3C). Fig. 3D depicts a Western blot demonstrating that the increased expression of monocyte markers is also coupled to up-regulation of monocyte transcription factors IRF8, KLF4 and MAFB, that have already been described as miR-130a targets [8].

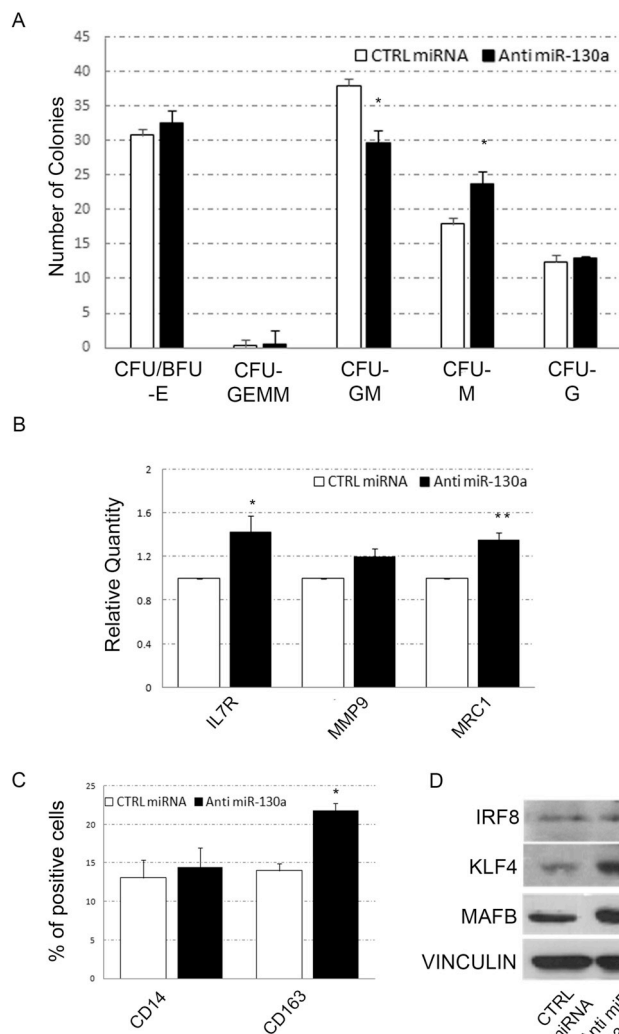
**Effect of specific PNA targeting miR-130a on haematopoietic differentiation.** Since miR-130a expression is de-regulated in AML, we tested the efficacy of PNA targeting this microRNA, to evaluate PNAs’ efficacy in inhibiting miR-130a expression and their effect on



**Fig. 2.** Effect of ectopic expression of miR-130a in human CD34<sup>+</sup> haematopoietic stem cells. (A) Methylcellulose assay showing that miR-130a ectopic expression determines an increase of CFU-GM coupled to a decrease of CFU-M. The number of colonies on the Y axis derives from at least three independent experiments. Results are represented as mean  $\pm$  S.E.M. values and asterisks indicate statistically significant results ( $P < 0.05$ ). (B) QRT-PCR showing the expression of a pattern of monocyte-related genes in HSCs following miR-130a ectopic expression. Mean variations of mRNA expression levels, deriving from at least three independent experiments, are reported in the y axis as relative fold-change (relative quantity). Results are represented as mean  $\pm$  S.E.M. values and asterisks indicate statistically significant results ( $P < 0.05$ ). (C) Flow cytometry analysis showing the percentage of cells positive to CD14 and CD163 respectively, following ectopic expression of miR-130a in CD34<sup>+</sup> cells. The percentage of positivity shown on the y axis derives from at least three independent experiments. Results are represented as mean  $\pm$  S.E.M. values and asterisks indicate statistically significant results ( $P < 0.05$ ). (D) Western blot analysis performed on monocyte differentiation related transcription factors following ectopic expression of miR-130a in CD34<sup>+</sup> stem cells. This analysis was performed on total protein extracts and normalized with Vinculin. Analyzed proteins are indicated on the left.

haematopoietic differentiation. Fig. 4A summarizes the results of a methylcellulose assay performed on HSCs untreated or treated respectively with scramble, degenerate and PNA-a130a. Results show that the decrease of CFU-GM and the increase of CFU-M comparable to that observed by anti-miRNA treatment (Fig. 3A) is observed only by anti-miR-130a PNA treatment. Western blot analysis shown in Fig. 4B suggests that up-regulation of monocyte transcription factors IRF8, KLF4 and MAFB occurs only in HSCs treated with the specific PNA targeting

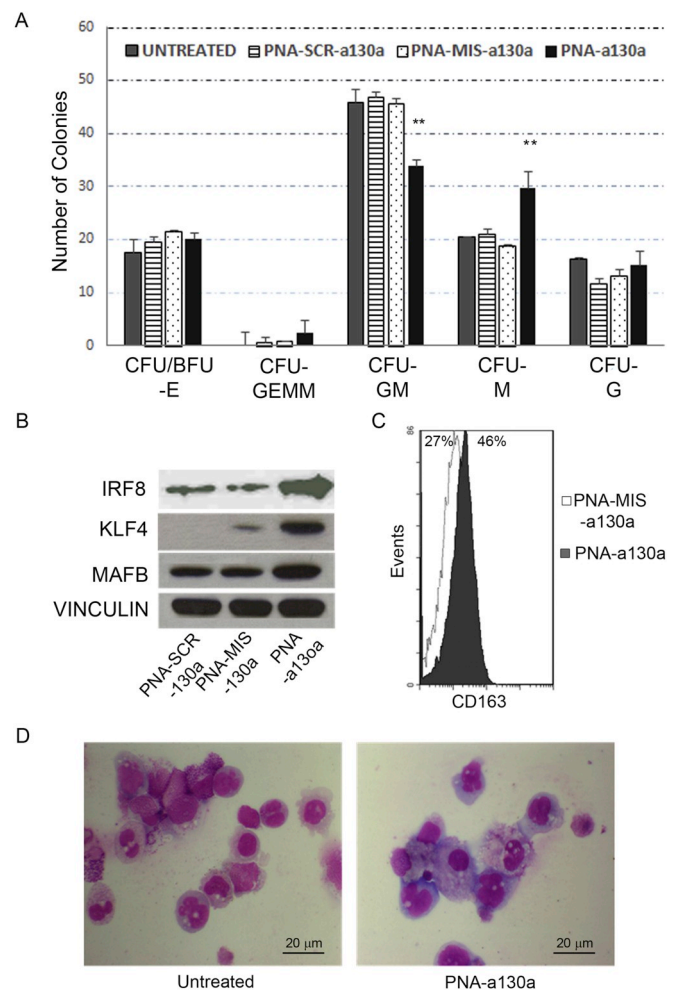




**Fig. 3.** Effect of miR-130a silencing in human CD34<sup>+</sup> stem cells. (A) Methylcellulose assay showing that miR-130a silencing determines a decrease of CFU-GM coupled to an increase of CFU-M. The number of colonies on the Y axis derives from at least three independent experiments. Results are represented as mean  $\pm$  S.E.M. values and asterisks indicate statistically significant results ( $P < 0.05$ ). (B) RT-PCR showing that miR-130a silencing in CD34<sup>+</sup> cells determines the up-regulation of monocyte-related genes IL7R, MMP9 and MRC1. Mean variations of mRNA expression levels, deriving from at least three independent experiments, are reported in the y axis as relative fold-change (relative quantity). Results are represented as mean  $\pm$  S.E.M. values and asterisks indicate statistically significant results ( $P < 0.05$ ). (C) Flow cytometry analysis showing the percentage of cells positive to CD14 and CD163 respectively, following silencing of miR-130a in CD34<sup>+</sup> cells. The percentage of positivity shown on the y axis derives from at least three independent experiments. Results are represented as mean  $\pm$  S.E.M. values and asterisks indicate statistically significant results ( $P < 0.05$ ). (D) Western blot analysis performed on monocyte markers following silencing of miR-130a in CD34<sup>+</sup> stem cells. This analysis was performed on total protein extracts and normalized with Vinculin. Analyzed proteins are indicated on the left.

miR-130a. Moreover, cells where miR-130a was silenced by PNA treatment, show higher positivity to CD163 differentiation marker than the control, as shown by flow cytometry analysis (Fig. 4C). Fig. 4D depicts a May-Grunwald staining of HSCs untreated or treated with PNA-a130a. The picture shows a randomly selected field suggesting that the cell population not expressing the miRNA is richer in mono/macrophages than the control.

**Effect of specific PNA targeting miR-130a on different leukemic cell lines.** Since we hypothesized that PNAs might be a therapeutic tool

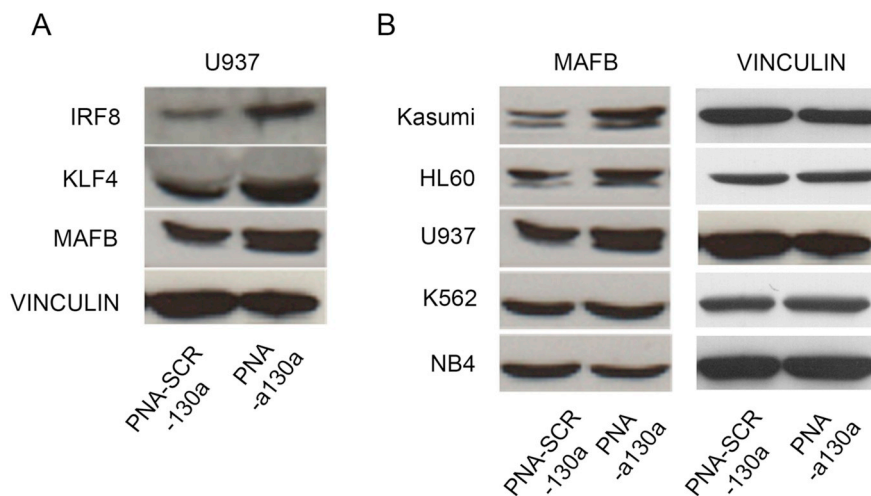


**Fig. 4.** Effect of specific PNA targeting miR-130a on haematopoietic differentiation. (A) Methylcellulose assay performed on HSCs untreated or treated with scramble (PNA-SCR-a130a), degenerate (PNA-MIS-a130a) or miR-130a targeting PNA (PNA-a130a). The number of colonies on the Y axis derives from at least three independent experiments. Results are represented as mean  $\pm$  S.E.M. values and asterisks indicate statistically significant results ( $P < 0.05$ ). (B) Western blot analysis showing the expression of IRF8, MAFB and KLF4 in HSCs treated with scramble, degenerate or specific PNA (PNA-a130a). This analysis was performed on total protein extracts and normalized with Vinculin. (C) Flow cytometry analysis showing the percentage of HSCs positive to CD163, following treatment with degenerate (PNA-MIS-a130a) or specific PNA (PNA-a130a). (D) May-Grunwald staining of HSCs untreated or treated with PNA-a130a.

targeting micro-RNAs, we analyzed its effect, from a molecular point of view, on selected leukemic cell lines. In particular we performed a Western blot targeting MAFB, IRF8 and KLF4 in U937 cells that are responsive to monocyte differentiation induction by phorbol esters (PMA) and VD. Fig. 5A shows that following PNA treatment the three genes are all upregulated compared to the control. Then we analyzed MAFB induction in several other cell lines responsive or unresponsive to monocyte differentiation inducers. Results are shown in Fig. 5B and suggest that MAFB is induced by PNA treatment only in cell lines that respond to monocyte differentiation inducers (Kasumi, U937 and HL-60) and not in un-responsive cell lines (K562 and NB4) suggesting that targeting miR-130a might be useful in specific forms of AML.

#### 4. Discussion

TFs and miRNAs act in concert to regulate gene expression during hematopoietic differentiation: TFs regulate the expression of miRNA



**Fig. 5.** Western blot analysis showing the induction of monocyte specific transcription factors in cell lines treated with miR-130a targeting PNA. Panel A shows the induction of MAFB, KLF4 and IRF8 in the U937 cell line. Panel B shows the induction of MAFB in Kasumi, U937, HL-60, K562 and NB4 cell lines. These analysis were performed on total protein extracts and normalized with Vinculin.

genes, and they are directly targeted by miRNAs. miRNAs represent an important axis of regulation in HSCs: they play a critical role in the control of stem/progenitor, lymphoid, myeloid, erythroid and megakaryocytic biology, and in the immune function of these cell lineages [25]. Moreover, individual miRNAs and miRNA signatures have been associated with specific hematological malignancies.

Acute myeloid leukemia is characterized by an accumulation of granulocytic or monocyte precursors in bone marrow and peripheral blood. miRNA patterns have been correlated with cytogenetic and molecular subtypes of AML [26]. Comparing the microRNA expression profiles of M1 and M5 AML, Lutherborrow et al. [8] found higher levels of miR-181a/b/d, miR-130a, miR-135b, miR-146a/b and miR-663 in M1 AML samples. Interestingly, the targets of these miRNAs are key transcription factors involved in monocyte/macrophage differentiation: KLF4, MAFB, HOXA10 and IRF8.

In order to evaluate the role of miR-130a during monocyte differentiation, we analyzed the expression of miR-130a during myelopoiesis. The data obtained showed that miR-130a is significantly higher in myeloblasts. These results correlate well with the fact that miR-130a-3p targets HOXA10, IRF8, KLF4 and MAFB [8] that are considered master regulators of monocyte differentiation [27–32].

To confirm the decrease of the two studied miRNAs in monocytopoiesis, we stimulated HSCs with Vitamin D, that is a strong inducer of monocyte-macrophage stimulation. As expected, a decrease of miR-130a expression was observed, further suggesting that down-regulation of miR-130a is necessary to trigger monocyte differentiation. These data also suggest that miR-130a misregulation might contribute to aberrant monocytopoiesis.

Gain- and loss-of-function experiments confirmed that by acting on miR-130a expression it is possible to impinge on monocyte differentiation. Since deregulation of this miRNA has been described in AML, we hypothesized that they could represent a therapeutic target for certain disease subgroups. For this reason, we decided to verify the efficacy of peptide nucleic acids (PNA) to silence the expression of miR-130a in HSCs. PNAs have very important properties: thermal stability, strong binding to RNA, capacity to form stable triple-helical complex, greater specificity of interaction, resistance to nucleases and proteases and some recent modifications, including the incorporation of positively charged lysine residues, have shown improvements in solubility. The results obtained show a strong uptake of PNA-a130a in absence of transfection reagent in hematopoietic stem cells that are hard to transfect. The analysis performed at day 14 of culture confirmed that PNA is very stable and that a single treatment is sufficient to have an anti-miR effect.

The evaluation of monocyte differentiation in HSCs treated with anti-miR-130a PNA, documents a higher increase of monocytopoiesis

when compared with the samples treated with anti-miR molecules, demonstrating that the biological effect of PNAs in modulating miRNA expression is strong and very stable. This last set of data highlights that the modulation of miR-130a expression is necessary to a correct myeloid differentiation, and throws the basis for a possible future use of PNA-a130a as therapeutic agent to restore a correct gene expression in hematological malignancies like AML. This latter hypothesis is reinforced by the observation that anti-miR-130a treatment is capable of inducing the expression of the monocyte master gene MAFB in cell lines responsive to monocyte differentiation inducers, suggesting its efficacy in specific subsets of the disease.

#### Author disclosure statement

All the authors certify that they have NO competing financial interest and conflict of interest in the subject matter or materials discussed in this manuscript.

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